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Cellular Control of the Synthesis and Activity of the Bacterial Luminescent System[1]

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In bioluminescent bacteria growing in shake flasks, the enzyme luciferase has been shown to be synthesized in a relatively short burst during the period of exponential growth. The luciferase gene appears to be completely inactive in a freshly inoculated culture; the pulse of preferential luciferase synthesis which occurs later is the consequence of its activation at the level of deoxyribonucleic acid transcription which is attributed to an effect of a "conditioning" of the medium by the growing of cells. Although cells grown in a minimal medium also exhibit a similar burst of synthesis of the luminescent system, the amount of synthesis is quantitatively less, relative to cell mass. Under such conditions, added arginine results in a striking stimulation of bioluminescence. This is attributed to a stimulation of existing patterns of synthesis and not to induction or derepression per se.

The control of the appearance of luminescence during the growth cycle is a spectacular but poorly described and ill-understood feature of the bioluminescent bacteria. The matter of interest in this paper is that the growth of the bacteria and the development of luminescence do not occur in concert in liquid cultures. The development of light emission is delayed and starts only during the middle of the logarithmic phase of growth. Its rise is then far more rapid than is the increase in cell mass during that time.

The phenomenon is illustrated in the plots of Fig. 1 and 2, which present the data in both exponential and linear form. The growth of the cells proceeds exponentially over the first few hours; in a complex medium, the doubling time at 25 C is about 25 min. But the in vivo bioluminescence of the cells does not increase during that time; it actually decreases and begins to rise only at a later time. Its rise is then very quick; the light output may double about every 4 or 5 min. At its peak, the luminescent enzyme (luciferase) constitutes between 2 and 5% of the soluble protein of the cell (6).

The experiments described in this paper were carried out in an attempt to better understand this phenomenon, especially the conditions which control the synthesis of luciferase. We concluded that in a freshly inoculated culture the gene controlling luciferase synthesis is inactive and that neither luciferase nor its messenger is being synthesized. The onset of in vivo luminescence is hypothesized to occur by means of an activation of the luciferase gene resulting in the stimulation of messenger ribonucleic acid (mRNA) synthesis, followed by luciferase synthesis. Since the phenomenon occurs without external intervention, it must be attributed to a conditioning of the medium effected by the growing cells (9). We have referred to the phenomenon as "autoinduction."

The behavior of cells growing in a minimal medium has important similarities to that of cells in a complex medium, but there are also significant differences. After inoculation into a fresh medium, there is, similarly, a period during which no luciferase synthesis occurs, followed by a pulse of relatively rapid synthesis. The autoinduction phenomenon is thus basically similar to that which occurs in complex medium, but it differs with regard to the extent of autoinduction, i.e., in the amount of synthesis which results. Less synthesis of both the luciferase and other cofactor(s) occurs, so that the in vivo bioluminescence appears very dim indeed.

Coffey reported that the bioluminescence of cells growing in minimal medium can be greatly stimulated by the addition of arginine (1). Although this effect was referred to as induction, we found that the arginine acts instead by simply enhancing the autoinduction, stimulating the synthesis of both luciferase and the other factor(s)

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to higher levels. On the basis of cell mass, the luciferase content of such cells is similar to the content of cells grown in complex medium.

MATERIALS AND METHODS

Photobacterium fischeri strain MAV has been described previously (8) and is designated as the wild type. Mutants were derived from this strain by mutagenesis with nitrosoguanidine (K. H. Nealson, 1969, Ph.D. Thesis, University of Chicago).

Minimal medium was similar to that of Farghaly (3), containing 30 g of NaCl, 7 g of $Na_2HPO \cdot 7H_2O$, 1 g of KH_2PO_4, 0.5 g of $(NH_4)_2PO_4$, 0.1 g of $MgSO_4$, and 3 ml of glycerol per liter of distilled water. Complex medium was prepared by adding 0.5 g of yeast extract (Difco), 5 g of peptone (Difco), and 3 ml of glycerol to 1 liter of sea water. For solid media, 11 g of agar (Difco) were added per liter.

The experiments were carried out in liquid shake cultures at 25 to 27 C. Since the amount of luminescence varies to some extent with the amount of aeration, care was taken to keep the shaking conditions as similar as possible in different experiments. Using 300-ml flasks with a culture volume of 75 ml, the generation time in the complex medium was approximately 25 min, whereas in the minimal medium it was approximately 250 min. For large experiments similar conditions were found to pertain by using a 500-ml culture volume in 2-liter flasks.

The light measuring equipment employed a photomultiplier tube (1P21) enclosed in a lighttight chamber to which the sample could be exposed by a shutter mechanism (7). The tube was operated at 1,000 v, and, after appropriate amplification, the output was monitored on an Esterline-Angus Speed Servo recorder. Light intensity, as established by the standards of Hastings and Weber (7), is expressed in quanta/sec.

Measurements on the cells during growth were carried out by using portions removed from the shake flask at the times indicated. Samples were discarded after use. Cell density was determined at 660 nm by using test tubes in a Coleman Jr. Spectrophotometer and is expressed in the optical density units of that instrument. These measurements were accompanied by viable cell counts which were carried out by plating on the complex medium after appropriate dilution. Well aerated cells emit continuously at a relatively constant intensity, and the in vivo bioluminescence was measured with 1 ml of such a sample. Cyanide (0.015 M) and aldehyde, which stimulated luminescence under certain conditions, were added (in sea water solutions) by injection from a syringe to the vial positioned in front of the phototube during the actual light measurement.

In vitro determinations of luciferase were carried out by measurements of its activity in extracts of cells. Culture portions were harvested on membrane filters (Millipore Corp.) or by centrifugation at 15,000 × *g* for 10 min. Extraction was accomplished by lysis at low ionic strength; after quick freezing and thawing, luciferase was extracted in 0.01 M ethylenediaminetetraacetic acid (EDTA) (*p*H 7.0) with 10^{-3} M dithiothreitol added. The luciferase assay was carried out as previously described (8), by measuring the initial maximum light intensity (I_0) upon mixing with 1 ml of reduced flavine mononucleotide ($FMNH_2$; 5×10^{-5} M) in the presence of aldehyde (*n*-decanal) and oxygen. The overall reaction may be represented by the following equation:

$$FMNH_2 + \text{luciferase} + O_2 + RCHO \rightarrow \text{light} + \text{products}$$

The reaction mixture before $FMNH_2$ addition contained 0.1 ml of the cell extract (luciferase), 0.1 ml of decanal solution and 1 ml of 0.1% bovine serum albumin in 0.075 M phosphate buffer (*p*H 7.5). Reactions were carried out at 22 ± 2 C. In all experiments, the luciferase activity of the extract is expressed as the activity obtained from 1 ml of the cell culture.

Chemical reagents were of analytical quality where available. Flavine mononucleotide (FMN), amino acids, and chloramphenicol were obtained from Sigma Chemical Co., St. Louis. Puromycin dihydrochloride was purchased from Nutritional Biochemicals, Cleveland, Ohio. Rifampin was obtained from CIBA Pharmaceutical Co., Summit, N.J. Platinized asbestos was obtained from E. H. Sargent and Co. Stock solutions of decanal (K and K Laboratories, Plainview, N.Y.) were prepared by sonic disruption of a 0.1-ml portion in 10 ml of distilled water. Solutions for use were diluted 1 to 100 and prepared fresh each day.

Antibody to luciferase was produced in rabbits by using purified luciferase as the immunogen (suspended in Freund's adjuvant at a concentration of 1 mg/ml). The primary 0.5-ml subcutaneous injection made in the neck was followed 21 days later by an identical secondary injection. Serum was harvested 10 days later and found to contain a high titer of antiluciferase. Since luciferase is enzymatically inactive after reaction with antiluciferase, the quantity of luciferase and antigenically cross-reacting material (CRM) could be measured by determining the amount of antiluciferase required to give inhibition. This was done using a modification of the method of Tsuji and Davis (11).

RESULTS

In a freshly inoculated culture, light emission does not begin to increase until a considerable amount of growth has occurred (Fig. 1 and 2). Several explanations have been suggested to account for this. The explanations can be grouped in four categories, depending on whether the control involved is hypothesized to operate at the level of substrate control, enzyme activation, translation, or transcription. It has been proposed that the lag is due to an inhibitor in the medium which is removed by a "conditioning" of the medium during growth (9). Although this hypothesis seems inadequate to explain the experimental data which we have obtained, it could be accomodated into any one of the four categories proposed.

Let us consider first the possibility of substrate control. It is possible that the failure of cells to emit light during the early stages after inoculation might simply be due to a competition for electrons (2, 4, 10). Since $FMNH_2$ (the substrate for the bioluminescent oxidation) derives from the reducing power of the cell, it is possible that a control mechanism exists which channels electrons during growth. The requirements for reducing power for adenosine triphosphate generation and biosynthesis might then be preferentially accomodated during the early stages of growth in a shake flask. A prediction of this theory is that active luciferase is synthesized at all times along with other enzymes and cellular constituents but that it is inactive because of the unavailability of its substrate. The experiment of Fig. 1 refutes this particular hypothesis because there is no increase in the total extractable luciferase activity; during the first 3 to 4 hr of growth it remains virtually constant. This might be due to a precise balance between synthesis and breakdown; alternatively, the luciferase present in the cells at the time they are inoculated into the fresh medium (zero hours) might be distributed without loss among the progeny during growth. As will be described later, the latter alternative is supported by experiments with inhibitors of protein synthesis.

Figure 1 also shows that the spectacular rise of the in vivo luminescence can be attributed to and closely parallels the appearance of extractable active luciferase. At the same time, it is evident and obligatory that most or all other enzymes, unlike luciferase, are synthesized in concert with growth. This has been shown to be true for glucose-6-phosphate dehydrogenase (K. H. Nealson, 1969, Ph.D. Thesis, University of Chicago), and also for a flavine reductase (W. C. Duane, 1969, Ph.D. Thesis, University of Illinois, Urbana).

Another prediction of this first hypothesis is than an inhibitor such as cyanide, which blocks electron flow through cytochromes to oxygen, should greatly stimulate luminescence during the "eclipse" period but not at times when in vivo luminescence is maximal. Actually, cyanide is known to stimulate luminescence under low oxygen tension (K. L. Van Schouwenburg, 1938, Ph.D. Thesis, Delft, Holland; reference 4) implying that a competition for electrons does exist under these conditions.

We therefore measured the effect of cyanide upon luciferase at various times during growth and found a differential stimulation (Fig. 1 and 3). But the stimulatory effect evidently relates to the interesting and previously unexplained decrease in the in vivo luminescence which occurs

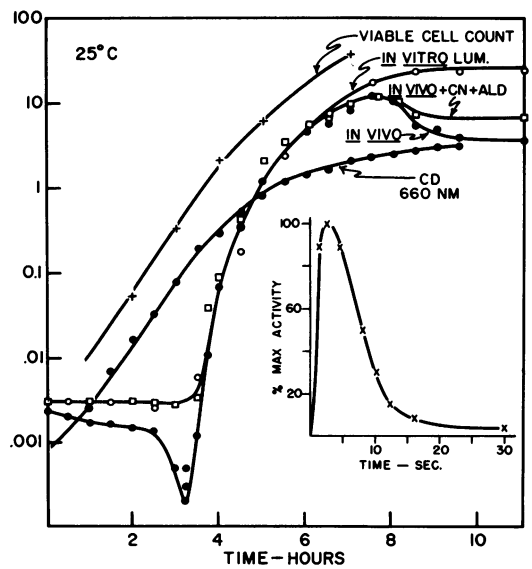

FIG. 1. *Time course of growth and luminescence of P. fischeri MAV cells in complex medium. The ordinate values, plotted on a logarithmic scale, should be multiplied by the factors specified to obtain the experimental values. Growth was measured both by viable cell count (10^8 cells/ml) and cell density (CD; ×1). Luminescence (2×10^{10} quanta per sec per ml) was measured both in vitro (the activity of the extractable luciferase) and in vivo. In the latter case, the luminescence was also measured subsequent to the addition of 0.015 M potassium cyanide and aldehyde (decanal). These additions stimulated light emission during the first and last few hours, but not during the intervening hours when active luciferase synthesis was taking place. The kinetics of the response to aldehyde are illustrated in the inset.*

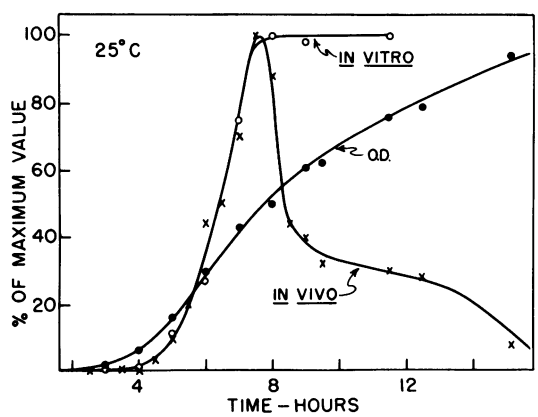

FIG. 2. *Experiment of Fig. 1 plotted on a linear scale, better illustrating the "pulse" nature of the luciferase synthesis and in vivo luminescence. The ordinate values were all normalized to the maximum and plotted as the per cent of that value.*

during the first few hours. Thus, electron channeling does occur during this period, and it can be redirected, presumably by cyanide inhibition of respiration.

At the same time, the stimulation by cyanide never results in an in vivo activity exceeding that which was present at the time of inoculation, thereby supporting the thesis that there is no increase in the quantity of the luciferease during the eclipse period. Actually, cyanide alone fails to restore the original in vivo activity, but if aldehyde (*n*-decanal) is added after the cyanide stimulation has come to a steady state (about 1 min), an increase occurs to a level of light emission comparable to that which the cells exhibited at the time of inoculation (Fig. 1 and 3).

In summary, the decrease in the in vivo activity during the eclipse period is not due to the loss of luciferase, but to a combination of two other factors: (i) substrate (electron) deficiency which can be restored by cyanide, and (ii) the loss or dilution out of the endogenous cellular aldehyde or aldehyde factor. With regard to the phenomenon under consideration, namely the apparent delay in the synthesis of new luciferase, the fact that there is no increase in luminescence (either in vivo or in vitro) during the first few hours cannot be attributed to substrate control.

The second possibility is that luciferase synthesis actually does accompany cell growth, but that the enzyme is produced in an inactive form requiring only some specific activation step. On the assumption that the hypothetical zymogen is antigenically reactive with antiluciferase, this hypothesis predicts that antigenically CRM would be produced during the early phases of growth and that its quantity would be proportional to cell mass. The titer of CRM (as determined by activity inhibition with anti-luciferase) closely parallels the extractable activity (Fig. 4).

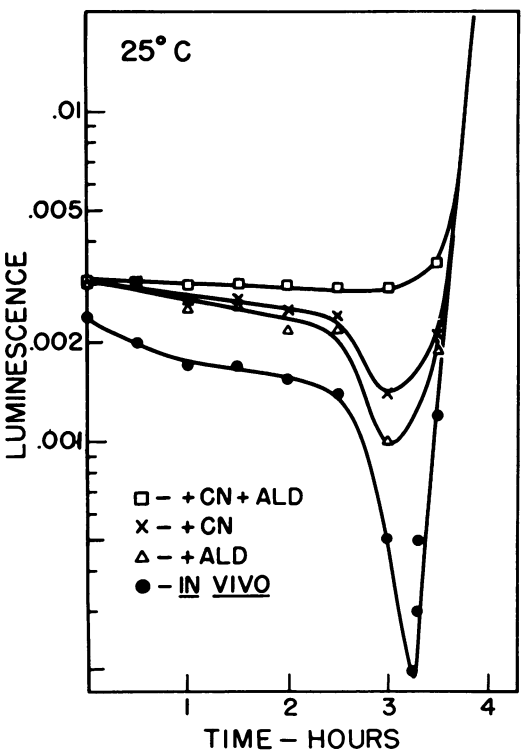

FIG. 3. *Details of the in vivo luminescence of the experiment of Fig. 1 during the first few hours, showing the stimulation by cyanide and aldehyde, individually and together. The luminescence of 1-ml samples of the cells was measured at the times indicated, and then 0.1 ml of either 0.015 M KCN in sea water or a saturated decanal solution was injected. When both were added, the cyanide was added first, and after the maximal response (~1 min) the aldehyde was added. The response to aldehyde was rapid and the luminescence then declined rather quickly (inset, Fig. 1).*

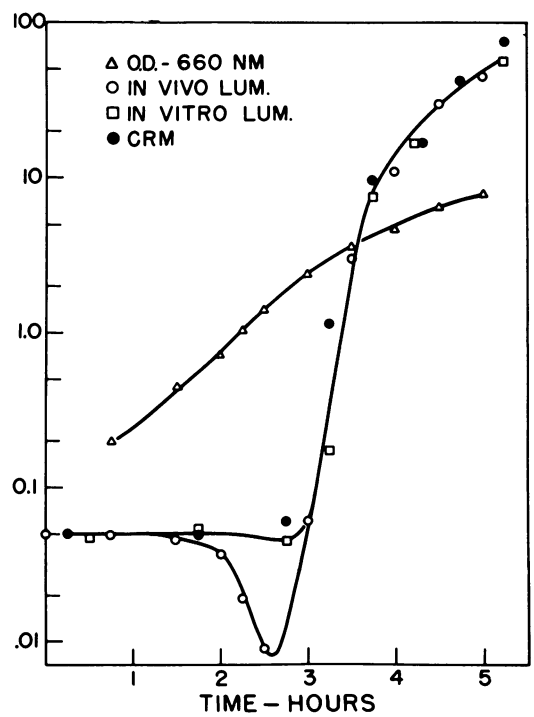

FIG. 4. *The kinetics of the appearance of antigenically cross-reacting material (CRM) to antibody prepared against highly purified MAV luciferase (8), showing that it closely parallels the activity of extractable luciferase. Culture and growth conditions similar to those of the experiment of Fig. 1. Values of ordinates also the same as in Fig. 1; the CRM values were normalized.*

This hypothesis has thus not been supported by experiment.

The third possibility to consider is that translational control exists. It might be supposed that the mRNA for luciferase is uniformly synthesized as growth proceeds but is not active; that it accumulates and then is called into activity during a specific phase of growth. A prediction of this hypothesis is that the rapid rise of luminescence should be sensitive to inhibitors of protein synthesis (chloramphenicol, puromycin, and kanamycin) but not to those which block synthesis of mRNA (rifampin or actinomycin D). This is not the case (Fig. 5 and 6); as measured by its extractable activity, the synthesis of luciferase is blocked by both types of inhibitors, thus supporting the hypothesis that the phenomenon is due to a control mechanism which operates at the level of transcription. However, the luciferase present at the time the inhibitor is added persists as extractable activity.

The in vivo luminescence is also very sensitive to the inhibitors (Fig. 5 and 6). Actually, not only is the further increase in luminescence blocked; a marked decrease in the bioluminescence occurs after the addition of either one of the inhibitors. Since the extractable luciferase activity does not decrease, this cannot be attributed to luciferase inactivation. An interesting feature is that the decrease is more pronounced when inhibitor is added after autoinduction than if added before. When added afterwards, there is a precipitous decline in bioluminescence to levels even below that of parallel cultures to which the inhibitor had been added at an earlier time. But as noted above, the extractable luciferase remains constant. The in vivo luminescence of such inhibited cells could not be stimulated by the addition of cyanide and aldehyde. Thus, the decline after the addition of inhibitors cannot be attributed to the same factors which are responsible for the spontaneous transient eclipse of luminescence which occurs in newly inoculated cultures. There is no apparent explanation available at the present time to account for the in vivo inhibition.

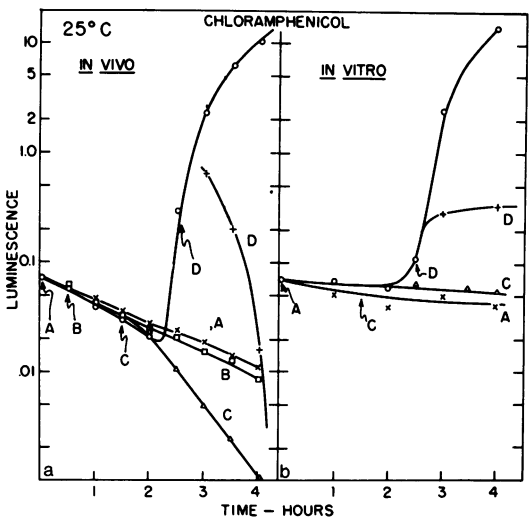

FIG. 5. *Effect of chloramphenicol on the in vivo luminescence (a) and the in vitro luciferase levels (b) when added at various times, after inoculation, A, B, C, and D, as indicated by arrows. The culture and growth conditions were similar to those of experiment of Fig. 1. The control culture (○) was a 500 ml broth culture growing in a 2-liter flask. For each addition of chloramphenicol, 50 ml of culture was removed and placed in a 300-ml flask containing 5 mg of chloramphenicol. The letters A to D serve to identify the cultures corresponding to the various times of addition.*

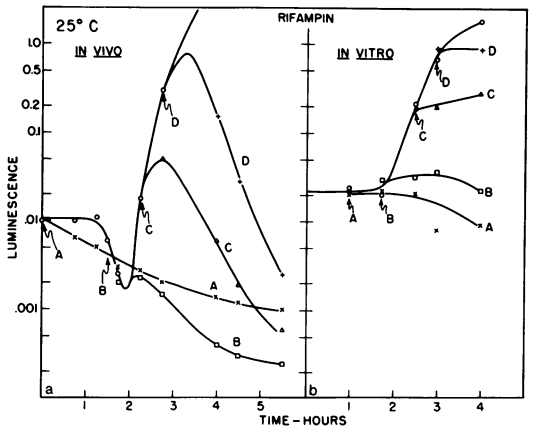

FIG. 6. *Effect of rifampin on the in vivo luminescence (a) and the extractable luciferase levels (b) when added at various times during the growth cycle. The experimental procedure was similar to that of the experiment of Fig. 5. A 500-ml broth culture growing in a 2-liter flask was used as the control. At each time indicated, 50 ml was removed and placed in a 300-ml flask containing 1 mg of rifampin.*

The experiments with rifampin and chloramphenicol thus indicate that both the luciferase messenger and the protein itself are synthesized late and at a rate faster than other cellular components, supporting the hypothesis that the control involved is indeed at the level of transcription.

Studies with cells grown in a minimal medium give additional support to this hypothesis (Fig. 7). Under these conditions, the cells not only grow much more slowly; they also emit much less light. However, when cyanide and aldehyde are added to the growing cells, it is possible to demonstrate that an appreciable amount of luciferase is actually being synthesized. Its pres-

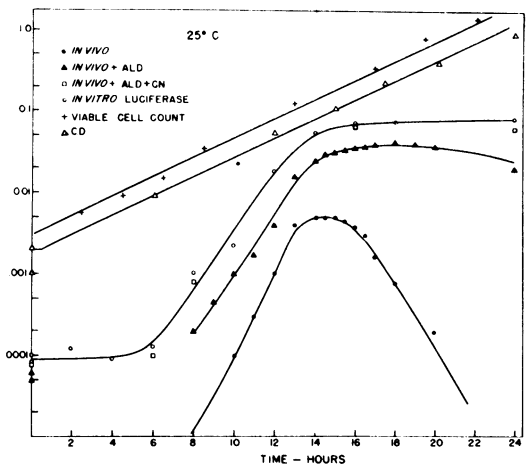

FIG. 7. *Time course of growth and luminescence of P. fischeri (MAV) cells in minimal medium. The ordinate values, plotted on a logarithmic scale, should be multiplied by the factors specified to obtain the experimental values. Growth was much slower than in complex medium and was measured both by viable cell count (10^8 cells per ml) and cell density (CD; ×1). The fact that in minimal medium there are more cells per optical density unit at 660 nm is attributed mostly to the fact that cells growing in minimal medium are considerably smaller (microscopic observations). Luminescence (2×10^8 quanta per sec per ml) was measured both in vitro and in vivo. Stimulation by either cyanide plus aldehyde or aldehyde alone shows that the in vivo emission, which was never very great, can be so stimulated at any and all times to give an accurate index of the luciferase content.*

ence and the kinetics of and quantity of its synthesis are measured by in vitro assays of luciferase activity in extracts.

The kinetics of luciferase synthesis are similar to those which occur in complex medium, in the sense that there is a period after inoculation when no luciferase synthesis occurs, followed by a burst during which its synthesis is more rapid than growth. This indicates that similar control phenomena occur in both media. A plot of the data of Fig. 7 on a linear scale is shown in Fig. 8.

The stimulation which occurs upon the addition of cyanide and aldehyde indicates that both the reduced substrate and the endogenous aldehyde are limiting in cells growing on a minimal medium, comparable to the situation which was observed to occur transiently after inoculation into fresh complex medium. However, in a minimal medium, there is a difference in that the luciferase is *never* fully active in vivo. The aldehyde factor and reducing power limit luminescence throughout.

Another reason for the failure of cells growing in a minimal medium to emit a very bright luminescence is related to the fact that the in vivo luminescence peaks at a time when the cell density is relatively low. Cells grown in minimal medium are smaller than those grown in complex, and the actual cell mass at the time of peak luminescence is quite low. Thus, the burst of luminescence phenomenon for cells growing in complex medium (Fig. 2) is even more dramatic in the case of cells growing in the minimal medium (Fig. 8). It truly comes and goes during the period of exponential growth.

Coffey (1) reported that luminous bacteria grown in a minimal medium are stimulated to emit much more light after the addition of arginine. Arginine appears to be unique in its ability to evoke this striking response and to act selectively upon the luminescent system; no stimulation of growth has been detected, even at high concentrations.

We found that the response is attributable not only to the synthesis of luciferase; after arginine addition the luminescence of the cells is no longer stimulable by aldehyde, indicating that it also involves the synthesis of that factor. Arginine does not alter the stimulability of luminescence by cyanide. The effect of arginine upon the in vivo luminescence, and its stimulability by aldehyde is shown in Fig. 9. It should be added that this response is fully blocked by inhibitors of RNA and protein synthesis. The data when inhibitors were added after the primary arginine addition (at 10 hr) have not been plotted; the results were closely comparable to those shown in Fig. 5 and 6.

The decline in the whole cell luminescence which occurs subsequent to the maximum can also be shown to be attributable to a loss of the aldehyde factor, as judged by stimulability (Fig. 9). Moreover, the decline in the in vivo luminescence can be reversed by a secondary addition of

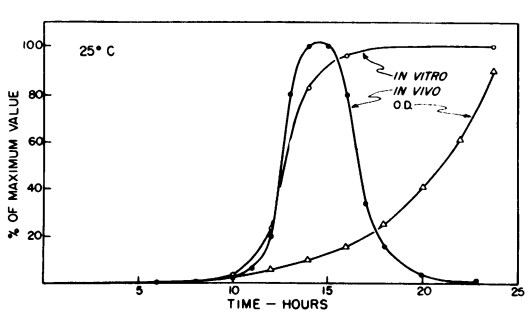

FIG. 8. *Experiment of Fig. 7 plotted on a linear scale showing that the "pulse" of luciferase synthesis occurs quite early in the growth, as estimated by the cell density. The ordinate values were normalized and are plotted as percent of that value.*

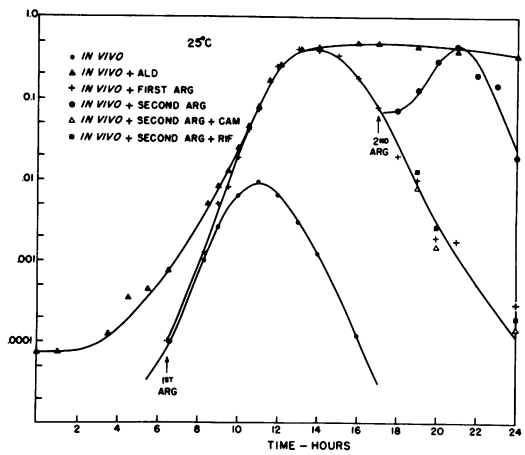

FIG. 9. *Stimulation by arginine of the in vivo bioluminescence of cells growing in a minimal medium. The addition of arginine (250 μg/ml, final concentration) at the time indicated results in an increase in the in vivo bioluminescence. This stimulation can be accounted for in part by the increased synthesis of luciferase (see Fig. 10) and in part to the synthesis of the aldehyde factor. After the arginine response the addition of aldehyde fails to stimulate. After the peak of luminescence, there is not only a cessation of synthesis of the luminescent system components; the in vivo luminescence itself dramatically declines and can again be stimulated by aldehyde. As shown, luminescence can then be stimulated by arginine; chloramphenicol and rifampin, when added along with arginine, again block this effect.*

arginine. This stimulation can be attributed to the synthesis of both luciferase and aldehyde factor, and again the response is blocked by inhibitors of RNA and protein synthesis (Fig. 9).

In spite of the relatively dim emission of cells growing in minimal medium, the amount of luciferase synthesized after the autoinduction is quantitatively equal to about 5% (on the basis of cell mass or protein) of that which is formed in a complex medium. On the same basis, the amount of luciferase formed after arginine stimulation (Fig. 10) is about twice that which is synthesized in a complex medium (Table 1).

Coffey (1) had concluded (and with good reason) that the arginine stimulation constitutes a case of enzyme induction. When arginine is added to a culture which has already entered or completed the autoinduction phase, the response is rapid and similar to induction. But an authentic inducer should be equally effective, irrespective of when it is added. The experiments of Fig. 10 show that this is clearly not the case in this system, since the time at which the onset of luciferase synthesis occurs is essentially independent of both the time of arginine addition and the amount added. The arginine effect is essentially a potentiation of the autoinduction which occurs in its absence; it augments the amount but does not initiate the synthesis of luciferase and the aldehyde factor.

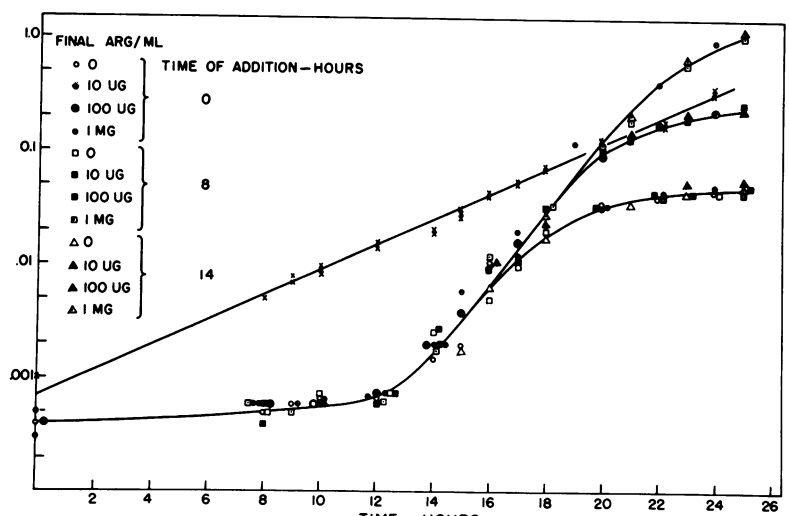

FIG. 10. *Effect of varying time and quantity of arginine addition upon the synthesis of extractable luciferase. Arginine was added at the three times indicated in the three concentrations noted (10, 100, and 1000 μg/ml, final). As measured by cell density (×) no differences were detected in the growth rates in the different experiments. The time at which the onset of luciferase synthesis occurred was also unaffected. More luciferase was synthesized in the flasks containing the higher arginine concentrations, but the time of addition of the arginine had no influence. Luciferase activity is expressed as cells extracted per ml. Ordinates: cell density (×1) and luminescence (2 × 10^{10} quanta/sec).*

TABLE 1. *Levels of in vivo luminescence and extractable luciferase obtained during growth on complex, minimal, and arginine-supplemented minimal media*[a]

Media	Luminescence intensities (quanta per sec per ml)	Cell density at 660 nm	Viable count cells per ml	Protein in crude extract (mg/ml)	Quanta per sec per cell	Quanta per sec per optical density unit	Quanta per sec per mg of protein
Complex							
In vivo	4×10^{12}	12.5	5×10^{9}	1.8	8×10^{2}	3×10^{10}	2.2×10^{12}
In vitro	4×10^{12}	12.5	5×10^{9}	1.8	8×10^{2}	3×10^{10}	2.2×10^{12}
Minimal							
In vivo	4.0×10^{7}	0.15	4×10^{8}	0.002	0.01	2.5×10^{8}	10^{10}
In vitro	5.0×10^{8}	0.15	4×10^{8}	0.002	0.1	3.1×10^{9}	1.3×10^{11}
Minimal + arginine							
In vivo	1.2×10^{10}	0.4	10^{9}	0.005	12	3×10^{10}	2.4×10^{12}
In vitro	2.0×10^{10}	0.4	10^{9}	0.005	20	5×10^{10}	4×10^{12}

[a] Measurements were made in each experiment at the time when the luminescence per cell was at a maximum. In minimal medium, for example, luminescence peaked at a low cell density and the cells were smaller (compare cell density with the viable cell count). In the last two columns, it is shown that, based on cell mass or extractable protein, the luciferase content of cells grown in minimal is about 5% of those grown in complex, whereas those grown in minimal plus arginine contain almost twice as much as the cells in complex.

DISCUSSION

On the basis of the experiments presented, we concluded that the control of the synthesis of bacterial luciferase is exerted at the level of transcription. In freshly inoculated cultures, the luciferase gene (or operon) is repressed or inactive; during the exponential period of growth its activation occurs, and luciferase is then rapidly and preferentially synthesized. We referred to this phenomenon as "autoinduction." The present experiments do not allow us to tell whether the control is negative or positive.

Kempner and Hanson (9) demonstrated that with cells inoculated into a preconditioned medium, i.e., a medium in which other cells had grown to a point of exponential light production, the characteristic lag in luciferase synthesis was abolished. We confirmed this observation and agree that the medium is indeed conditioned during the initial period of growth; however, from our experiments the exact nature of the conditioning is not clear.

With regard to the cellular control mechanism, we would expect that, if the control of luciferase synthesis does involve endogenous repressors and a derepression mechanism (or analogous positive control elements), mutants in which these systems are altered could be obtained. For example, if the repression of the luciferase gene were relieved (operator or repressor mutations), the mutant might appear as a "luciferase constitutive," in which the enzyme was synthesized at all times, in parallel with the growth of the cells. Screening for such mutants presents difficulties, since the in vivo luminescence also depends on the aldehyde factor. Nevertheless, it may be anticipated that such a mutant class will occur.

Actually, there appears to be a similar and simultaneous control on the synthesis of the different components of the bioluminescent system, i.e., luciferase and the aldehyde factor. This kind of coordinate control is reminiscent of that which occurs in an operon, and it is not unlikely that the bacterial bioluminescent system will be so classified.

The control of luciferase synthesis is in some ways analogous to a developmental phenomenon, since pulses of luciferase messenger occur only at a certain growth phase. The luminescent system represents an especially interesting and useful one for studying such phenomena, for both in vivo enzyme activity and the in vivo enzyme content can be continuously and instantaneously monitored. These experiments have illustrated the fact that intracellular enzyme may not always be fully active, showing that an extractable enzyme activity cannot always be reliably used to evaluate in vivo enzyme activity.

These studies have also served to clarify and emphasize the similarities and differences in the behavior of the cells in minimal and complex media, especially with regard to the autoinduction of the luminescent system. In both media the luciferase gene remains inactive during the first part of the growth cycle in shake-flasks and is then activated during the time when a burst of synthesis occurs. The fact that this same pattern occurs in both media indicates that the cellular

control phenomenon is essentially similar in the two cases.

The development of the luminescent system differs in the two media primarily in quantitative aspects. In the minimal medium, smaller quantities of both the luciferase and the aldehyde factor are found, thereby resulting in a drastic diminution of the in vivo light emission.

Arginine acts to relieve or remove the block, whatever it may be, which in minimal medium restricts the synthesis of the luminescent system, and is unable to act unless autoinduction has occurred. So long as arginine was believed to be an inducer of luciferase, it was difficult to understand why no effects of arginine addition could be demonstrated in the complex medium. In fact, the complex medium contains approximately 400 μg of arginine per ml. But since arginine has no effect in the minimal medium upon the duration of the lag period for luciferase synthesis, none would be expected in the complex medium. Moreover, since the components of the luminescent system are evidently synthesized in maximal quantities after autoinduction in the complex medium, no effects on the amount of synthesis would be anticipated with added arginine.

The experiments of Kempner and Hanson (9) were all carried out in a complex medium, and they hypothesized that the initial decrease in the in vivo luminescence should be attributed to the action of an inhibitor present in the medium and that conditioning involved its removal. It is difficult to reconcile this hypothesis with the fact that similar phenomena occur also in a minimal medium, in which the proposed inhibitor would probably not be present (or at least not identical). Moreover, the fact that the transient decline in the in vivo bioluminescence can be reversed by the addition of cyanide and aldehyde indicates that the luminescent system is in fact not inhibited during this period, as they had suggested, but is simply limited by the supply of reduced substrate and aldehyde.

The work of Coffey (1) was done with a bacterial strain which would utilize nitrate as the sole nitrogen source. He suggested that the failure of this organism to emit light on a minimal medium with nitrate, as well as the effect of arginine, might be a specific property of that strain. However, we observed that these phenomena are more general, occurring also with many different strains of luminous bacteria, including one which will not utilize nitrate. It will occur also with ammonia as the sole nitrogen source in a minimal medium, as illustrated by the experiments presented in this paper.

Although the nature of the control mechanism which acts on the luciferase gene is not known, its occurrence leads one to expect that the bioluminescence of these bacteria has some very special biological function. At the same time, the intact luminescent system does not appear to be essential for cells to grow and divide. This is evident when the cells are grown under conditions in which the repression is maintained semipermanently (e.g., by repeated subculturing). Luciferase is not synthesized under these conditions, and the cells will ultimately "grow out" of the luciferase by dilution. We also found that mutants which are deficient in luciferase are fully viable. This includes both mutants in which the protein itself is lacking, as judged by the absence of immunologically cross-reacting proteins and mutants which synthesize defective luciferase protein (Cline, *personal communication*).

McElroy and Seliger hypothesized (10) that the bioluminescent system in the bacteria is vestigial insofar as the light emission is concerned. However, we believe that a system controlled by truly vestigial genes would not persist with the characteristics which the luminescent system exhibits. For example, at its peak, luciferase occurs in large amounts; it constitutes about 5% of the soluble protein of the cell (6). Mutations in the luminescent system and in the luciferase occur readily, both in the laboratory and in nature (4). However, activity of and coordinate control over the system persists, suggesting that there has been a selection against mutations deleterious to the system. We thus believe that the luminescent system is in some way advantageous to the bacteria.

The precise nature of this function is unknown. The luminescent system might provide some metabolic advantage to the cell, independent of light emitted (5). On the other hand, the luminescence itself might be of positive value as, for example, in a symbiotic relationship in which bacterial luminescence is somehow selected for and utilized by the host. Certain marine fish possess specialized luminescent organs in which luminous bacteria are cultured and serve as the source of light (4). Such a role might constitute not only the biological basis for the occurrence and persistence of the luciferase gene(s) in bacteria; it might also relate to the unusual mechanism which controls the synthesis and activity of this luminescent system.

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